Mitochondrial Replacement Therapy By Nuclear Transfer In Human Oocytes

Background and Significance

Mitochondria, which are the "powerhouse" for most eukaryotic cells, are assembled with proteins encoded by the nuclear genome (nDNA) and mitochondrial genome (mtDNA) and are exclusively maternally inherited. mtDNA is a circular molecule consisting of 16,569 base pairs (bp) and encodes 13 polypeptides, as well as 22 transfer RNAs and two ribosomal RNAs (Wallace 1999). At least 1 in 5,000 people in the general population have one mtDNA mutation (Gorman et al., 2015), which can cause mitochondrial dysfunction and maternally inherited diseases (Schaefer et al., 2008; Wallace et al., 1988). When wild type and mutant mitochondria genomes co-exist (heteroplasmy), the severity of clinical symptoms is often associated with the level of heteroplasmy (Freyer et al., 2012). For example, mtDNA 8993 T>G mutation is associated with variable syndromes ranging from neuropathy, ataxia, and retinitis pigmentosa (NARP) (Tatuch et al., 1992) at the level of 70-90% mutation load to maternally inherited Leigh syndrome at level of 95% mutation load. Furthermore, when mtDNA 8993 T>G mutation load is less than 30%, a healthy child can be produced (Sallevelt et al., 2013), showing that mutation levels before displaying symptoms of disease depend on individual mutation tolerance thresholds.

Due to absence of effective treatment of mitochondrial disorders, the prevention of the transmission from mother to offspring is considered as the key management. Current options for prevention of transmission of mutated mtDNA include adoptions or use of donor eggs. Preimplantation genetic diagnosis (PGD) has been offered to detect pathogenic mtDNA mutation (Steffann et al., 2006, Craven et al., 2010) in order to select embryos with reduced mutation load. However, variation among blastomeres in a single embryo limits the effectiveness of PGD (Cree et al., 2008). Mitochondrial replacement techniques (MRTs) through nuclear transfer among

oocytes, which replaces mutated maternal mitochondria with the healthy mitochondrial of donor cells, has been proposed as a novel approach to prevent the transmission of mutant mtDNA from a carrier mother to her child at the gamete or zygote level. *In vitro* studies have reported successful fertilization and blastulation by MRT among human oocytes (Tachibana et al., 2013) and live birth of health offspring in non-human primate by MRT in oocytes (Tachibana et al., 2009). No individual nucleus with 46 XY from somatic cell will be transferred back to oocytes; therefore, cloning is not an ethical concern for MRT in human oocytes.

MRT technique could provide a new approach for a woman who carries mutant mtDNA to have a genetically related, healthy child without the concern of inheritance of the mtDNA disease in her offspring (Craven et al., 2010, Barritt et al., 2001). Our previous studies indicate MRTs can be carried out at germinal vesicle (GV), metaphase II (MII) stage of oocyte or pronuclear (PN) stage of zygote (Liu et al., 1999, 2000, 2003; Zhang et al., 2017a; Mykytenko et al., 2019). Furthermore, previous studies have shown that MII spindle nuclear transfer (SNT) was effective in preventing mtDNA transmission from oocytes to pre-implantation embryos (Tachibana et al., 2013; Zhang et al., 2017a; Mykytenko et al., 2019), SNT parthenogenetic embryonic stem cell line (Paull et al., 2013), monkey SNT offspring (Tachibana et al., 2009), and babies (Zhang et al., 2017a). Thus cytoplasmic replacement represents a promising technique to prevent the transmission of mutated mtDNA (Committee et al., 2016; Zhang et al., 2017a; Mykytenko et al., 2019).

Nuclear transfer technique gives rise to reconstituted oocytes with mitochondrial heteroplasmy, donor and recipient mitochondria in the cytoplasm (Kobayshit et al., 2008). The effect of mitochondrial heteroplasmy on meiosis resumption in human reconstructed oocyte is still not clear although a study in mice demonstrated that mitochondrial heteroplasmy of reconstituted

oocytes did not influence their in vitro maturation and preimplantation development (Kobayashi et al., 2008). The degree of mitochondrial DNA heteroplasmy, when mutated mitochondrial DNA is transported along with the nucleus to the reconstituted oocytes, could have implications on embryo development and ultimately the offspring (Yabuuchi et al., 2012). However, the level of acceptable mitochondrial DNA heteroplasmy in embryos created by nuclear transfer depends on the mitochondrial disorder of interest (Yabuuchi et al., 2012). In order to reduce the risk of transmission of mutated mitochondrial DNA into the reconstructed oocytes, it is necessary to completely remove mitochondrial DNA from the donor when nuclear transfer is performed (Craven et al., 2010). This remains a challenge in nuclear transfer technology although recent studies demonstrated promising results by using a new technique that completely eliminated mitochondrial DNA carry-over (Neupane et al., 2014).

Our group successfully performed SNT and PNT in mouse and human oocytes (Liu et al, 1999, 2000, and 2003; Zhang et al., 2017a), and sequential nuclear transfer GVT-SNT in human oocytes (Liu et al., 2017). The research team from Darwin Life achieved the first baby born from MRT - a healthy boy from a euploid male blastocyst containing minimal pathological mitochondrial carryover (5.7%; Zhang et al., 2017a). The baby boy also exhibited only 2.36-9.23% mtDNA mutation load in the tested neonatal tissues (Zhang et al., 2017a). In addition, the research team from Nadiya clinic has also recently achieved low-level heteroplasmy in embryos from MRT (Mykytenko et al., 2019). Specifically, 3 euploid male embryos exhibited 0.51 ± 0.02%, 2.96 ± 0.05% and 3.32 ± 0.06% mtDNA mutation load. Further study is mandatory and this clinical trial will continue to test the feasibility and safety of MRT.

Hypotheses and Objectives

I: Even though pathogenic mitochondria surrounding the patient nucleus will be carried over to the recipient, their level in the total mitochondria of the reconstituted oocyte is low. We hypothesize that transferred nucleus only carries a small amount of mitochondria to the recipient oocyte with nucleus removal. II. The embryo created from reconstituted mouse and human oocytes developed normally according to our previous data (Liu et al., 1999, 2000, 2003, and 2017; Zhang et al., 2017a; Mykytenko et al., 2019). We hypothesize that the embryo created from reconstituted human oocyte would develop normally before implantation.

Our objective is to evaluate the safety and efficacy of mitochondrial replacement therapy for women seeking to have a genetically related child whose prior IVF treatment has failed to result in a healthy pregnancy.

Methods and Design

Human oocytes at metaphase II (MII) phase or germinal vesicle (GV) phase will be collected. All patients enroll by their own consent to participate in the clinical trial.

I. Transfer of patient nucleus to donor ooplasm.

Nucleus from oocytes at GV (GVT), MII phase or from pronuclei from zygote stage after fertilization (NT group) will be removed by micropipette (Liu et al, 1999, 2000, and 2003) and placed into enucleated donor egg. At the same time donor nucleus will be placed into patient ooplasm for control (R-NT group). In order not to lyse the oocytes during removal of the patient nucleus or donor egg enucleation, membrane relaxants will be used such as Cytochalasin (Zhang et al., 2017a).

In order to maximize the number of embryos generated and reduce pathogenic carry-over, Polar Body Genome Transfer (PBGT) may also be conducted for the first polar body of the MII oocytes, in parallel to Spindle Nuclear Transfer (SNT). PBGT is only possible when MII oocyte exhibits a viable polar body, i.e. before the degeneration of the first polar body. PBGT from first polar bodies has been previously applied successfully in generating human blastocysts with no adverse impact on preimplantation development (Zhang et al., 2017b, Ma et al., 2017). Furthermore, previous research has evidenced that the PBGT technique results in undetectable carryover levels of patient mitochondria. The GV oocyte, MII spindle, MII polar body, or pronuclei will be combined with their retrospective donor cytoplasts using inactivated Cell Fusion Reagent GenomONE™-CF EX SeV-E (HVJ-E).

II. In Vitro Maturation (IVM).

GV oocytes will undergo IVM for 24h, or 48h until the mature MII phase is reached, at which point matured oocytes with a visible birefringent spindle will undergo SNT into *in vivo* matured donor MII oocytes (Liu at al. 2017, Liu et al., 2003). IVM is a standard procedure (Fesahat et al., 2017) which will be optimized for our conditions, by varying the culture drop size, the hours with cumulus cell attachment and cumulus cell co-culture, and the contents of the IVM media (Liu et al., 2018, Hirao et al., 2004). The conditions which result in maximum maturation rate in the control group will be used for in vitro maturation of GVs undergoing nuclear transfer. Every effort will be made to apply pre-selection of donors for donor MII oocytes in the SNT procedures Namely, the following criteria of will be applied for preselection of MII donors based on their cycle history: ≥18 retrieved MII oocytes with (i) minimum 80% normal fertilization rate, (ii) minimum 50%

rate of blastulation, and (iii) minimum 60% rate of euploidy. In order to maximize the number of embryos generated, Polar Body Genome Transfer (PBGT) may also be conducted for the first polar body of the in vitro matured MII oocytes, in parallel to Spindle Transfer.

III. Post fertilization rescue NT procedures.

After GVT and SNT/PBGT, the reconstituted oocytes will be fertilized with partner/donor sperm via intracytoplasmic sperm injection (ICSI). In case of abnormal fertilization (e.g. 3PN) on Day 1 after ICSI, microsurgical correction will be applied by removal of the extra pronucleus to result in 1 female and 1 male pronucleus (Kattera and Chen 2003). Identification of female pronuclei will be conducted by tracking the PN(s) from the Polar body extrusion on the Embryoscope/MIRI time-lapse recordings. In case of the reconstructed oocyte exhibiting only one female PN on Day 1, the oocyte will be vitrified for pronucleus transfer (PNT) procedures into donor oocyte fertilized with the patient's partner/donor sperm

IV. Preimplantation Embryonic Development post nuclear transfer.

The preimplantation embryonic development will be monitored after GVT, SNT/PBGT, or PNT by a senior embryologist. The embryonic development will be observed for fertilization, cleavage, and blastulation using standard morphological criteria (ALPHA Scientists In Reproductive Medicine; ESHRE Special Interest Group Embryology, 2011). At each checking point, the image or video will be taken. At blastocyst stage, the trophectoderm and inner cell mass (ICM) will be biopsied for aneuploidy analysis via Next

Generation Sequencing (NGS) and array-based Comparative genomic hybridization (aCGH).

We anticipate that the GVT, SNT/PBGT, or PNT would have no effect on the human preimplantation embryonic development.

V. Mitochondria heteroplasmy after nuclear transfer.

The ratio of donor to recipient mitochondria will be checked at blastocyst stage via previous protocols from our team (Zhang et al., 2017a). Similarly the differential expression of mitochondria heteroplasmy will be evaluated in trophectoderm and ICM. We anticipate that the ratio of donor to recipient mitochondria would change during the development of preimplantation embryos and heteroplasmy is differentiately expressed between trophectoderm and ICM. During the initial phase in this clinical trial, patients will be advised to restrict embryo transfer for intended pregnancy to only male embryos (Zhang et al., 2017a; Mykytenko et al., 2019). This restriction will serve as a precaution during the evaluation of the health of MRT babies and the prevention of inheritance of any changes which could appear in succeeding generations from female MRT babies. Furthermore in order to evaluate the MRT babies' health and heteroplasmy levels, whole mtDNA sequencing analysis will be conducted from whole blood and tissue samples from the neonate and in regular intervals until 18 years of age (Zhang et al., 2017a).

VI. **Materials**

Oocytes will be collected from patients with history of Mitochondria Disease. Such patients are enrolled in the study and through Informed Consents and doctor appointments with either Dr John Zhang or Dr Valery Zukin.

Data Management and Analysis

The results of the clinical trial will be analyzed and submitted in the form of manuscript(s), for independent peer-review in scientific journals.

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